Volume 230, number 1,2, 116–120 FEB 05710 March 1988

Glycogen synthase from human and bovine polymorphonuclear leukocyte

Immunochemical characterization and comparison to glycogen synthase from rat and rabbit muscle and liver cells

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Received 19 January 1988; revised version received 4 February 1988

Glycogen synthase from human and bovine polymorphonuclear leukocytes was purified to homogeneity. Rabbit antisera were raised against the two glycogen synthases and used for immunochemical analysis. Western blotting analysis showed that the subunit of glycogen synthase in crude homogenates of human and bovine leukocytes in both cases has an M_r of 85 000. The existence of a cross-reactivity between the two enzymes and the corresponding antisera demonstrates immunological similarities between bovine and human leukocyte glycogen synthase. In addition, both antisera recognize glycogen synthase in crude cellular extracts from rabbit and rat liver and from skeletal muscle. Leukocyte glycogen synthase, therefore, cannot be classified as either muscle (M-type) or liver (L-type) glycogen synthase and our results do not support the proposed immunochemical distinction between M- and L-type glycogen synthase.

Glycogen synthase; Polyclonal antibody; (Human leukocyte, Bovine leukocyte)

1. INTRODUCTION

Glycogen synthase (EC 2.4.1.11) is the rate-limiting enzyme in glycogen biosynthesis. Its activity is regulated by multi-site phosphorylation (review [1–4]).

The enzyme from rabbit skeletal muscle is the best-characterized glycogen synthase. A minimum of 10 serine residues are phosphorylated by at least 9 protein kinases [5–8], and many were found (in the case of the muscle glycogen synthase) to reside close to the N- and C-termini of the enzyme [9]. The leukocyte enzyme is less well characterized than the muscle glycogen synthase. The enzyme purified from human as well as from bovine polymorphonuclear leukocytes has a subunit size of 85 kDa [10,11], and on HPLC the tryptic peptides which are phosphorylated by cAMP-dependent protein kinase co-migrate with the tryptic phosphopeptides of the enzyme from rabbit skeletal muscle containing sites 1a, 1b and 2 [11]. One must expect, therefore, a strong homology in the amino acid sequences around sites 1, 1b and 2 in leukocyte and rabbit skeletal muscle glycogen synthase. Comparison of the amino-acid sequences surrounding phosphorylation sites recognized by cAMP-dependent protein kinase in rabbit liver and skeletal muscle glycogen synthase showed that

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Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HPLC, high performance liquid chromatography; M_r, molecular ratio; PMSF, phenylmethylsulphonyl fluoride; SDS, sodium dodecyl sulphate; TEMED, *N,N,N′,N′*-tetramethylenediamine; UDP-glucose, uridine 5′-diphosphoglucose

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

these enzymes are isozymes [12]. Immunoblot analysis has suggested that liver and muscle glycogen synthase are structurally different [13,14]. They were termed L- (liver) and M- (muscle) type, respectively. Using immunoblot analysis, Rulfs et al. [15] have identified a glycogen synthase subunit of 93 kDa from rat heart and liver homogenates and have suggested that a purified glycogen synthase with subunit size around 85 kDa is proteolysed. Thus partial proteolysis must be considered when phosphorylation is being studied, because terminal sites may be lost during purification of the enzyme. However, by measuring the subunit size of purified enzyme and glycogen synthase in fresh homogenates, one can eliminate this problem.

In this report, we describe the production and characterization of polyclonal antibodies against glycogen synthase from human and bovine polymorphonuclear leukocytes. Glycogen synthase in crude homogenates of human and bovine leukocytes were analyzed by Western immunoblotting. The molecular size was compared with the known M_r value of the subunit of purified leukocyte glycogen synthase. Furthermore, the antibodies against leukocyte glycogen synthase were used to test cross-reactivity with glycogen synthase in crude cell extracts from rabbit and rat skeletal muscle and liver.

2. MATERIALS AND METHODS

2.1. *Materials*

[U-^{14}C]UDP-glucose, [γ-^{32}P]ATP and [^{125}I]protein A were obtained from NEN Chemicals GmbH, FRG. Concanavalin A-Sepharose was a product of Pharmacia Fine Chemicals, Sweden. Nitrocellulose membrane (0.45 μm pore size) was from Millipore, USA. Benzamidine, soybean trypsin inhibitor, DTT, PMSF, and pre-stained molecular mass markers (SDS-7B) were from Sigma Chemical Company, USA. Acrylamide, bisacrylamide, and Nonidet P-40 were from BDH Chemicals, UK. TEMED and Coomassie blue G-250 were from Bio-Rad, USA. Semi-dry electroblotter was manufactured by Anchos, Denmark.

2.2. *Enzyme assay*

Glycogen synthase was assayed at 4 mM UDP-glucose in the absence (I-form) or presence (I + D-form) of 6.7 mM glucose 6-phosphate [16]. The ratio of independence is the ratio between the activities of the I-form and the I + D-forms. One unit (U) of glycogen synthase is the activity which incorporates 1 μmol of glycosyl units from UDP-glucose into glycogen per min at 30°C.

2.3. *Electrophoretic purification of the glycogen synthase subunit*

Bovine and human polymorphonuclear leukocytes were isolated from fresh blood samples as described [17,18]. Glycogen synthase I from bovine and human polymorphonuclear leukocytes were purified as described [11] including affinity chromatography on concanavalin A-Sepharose.

The glycogen synthase subunit which was used for immunization was isolated by extraction from SDS-polyacrylamide gels. Each lane contained 0.2 U (approx. 10 μg) glycogen synthase and ^{32}P-phosphorylated glycogen synthase as a marker. Autoradiograms of the wet gels were made and the gel sections containing the ^{32}P-labelled glycogen synthase subunit (85 kDa) were cut out. In order to extract the glycogen synthase subunit, the gel pieces were homogenized with an Ultra-Turrax homogenizer for 10 s in 1 ml of 0.9% NaCl and placed on a rocking table at 20°C overnight. The gel material was removed by centrifugation through glasswool and protein concentration was determined in the extract. The solution was lyophilized and stored at −70°C until used for immunization.

The protein concentration was determined by the Coomassie brilliant blue binding assay [19].

2.4. *Antibody production*

Rabbit antisera were raised against electrophoretically purified glycogen synthase subunits from human and bovine polymorphonuclear leukocytes. A subcutaneous injection of 50 μg of each antigen in Freund's complete adjuvant was followed by an intravenous injection of 50 μg antigen in 0.9% NaCl 30 days later. 50 ml blood was recovered 10 days after. The immunization and bleeding were repeated at 30-day intervals and the serum from the fifth bleeding was used in the immunoblotting experiments.

2.5. *Preparation of tissue extracts for immunoblotting experiments*

Leukocytes were isolated from fresh human and bovine blood samples as described [17,18]. A crude homogenate was obtained by ultrasonication as described [11]. Rats and rabbits were killed by cervical dislocation and muscle and liver tissue were excised immediately and homogenized in 40 mM EDTA (pH 7.0), 50 mM KF, and 5% glycerol using an Ultra-Turrax homogenizer. The homogenates were centrifuged at 17000 × g for 30 min at 4°C. The supernatants were assayed for glycogen synthase activity and the remaining supernatants were mixed with SDS-sample buffer [20] and heated at 95°C for 5 min. Amounts as indicated in the figure legends were electrophoresed into 7.5% SDS-polyacrylamide gels, and transferred electrophoretically to nitrocellulose membranes by a semi-dry electroblotting technique [21] modified into a one-buffer system as described [22].

3. RESULTS AND DISCUSSION

Glycogen synthase was purified from human and bovine polymorphonuclear leukocytes by affinity chromatography on concanavalin A-Sepharose followed by SDS-polyacrylamide gel electrophoresis. In each case, the enzyme subunit

was identified as a protein band at 85 kDa by incorporation of ^{32}P-phosphate catalysed by cAMP-dependent protein kinase. The respective proteins were extracted from the gels as described in section 2.2 and used to immunise rabbits. The immune response was followed by rocket immunoelectrophoresis of glycogen synthase in agarose gels containing the serum. Immune serum obtained after five immunisations was used in Western blotting analysis of glycogen synthase.

Crude protein extracts from human leukocytes were separated by 1-D SDS-polyacrylamide gel electrophoresis, electroblotted onto nitrocellulose and analysed with rabbit antiserum against human leukocyte glycogen synthase. Fig.1A shows the result of such a Western blot. A similar experiment in which antiserum against bovine leukocyte glycogen synthase was used is shown in fig.1B. Out of the vast diversity of proteins in the 17000 × *g* supernatants, both antisera recognize only one protein band of 85 kDa. The antisera also recognize an 85 kDa band in concanavalin A-Sepharose purified glycogen synthase from human and bovine leukocytes (not shown). Control experiments using sera from the rabbits before immunization did not show any cross-reactivity with crude leukocyte extracts (data not shown). The absence of protein bands at higher M_r indicates that the native subunit of human and bovine leukocyte glycogen synthase has an M_r of 85000, identical to the M_r value for the purified leukocyte glycogen synthase [10,11]. The antisera recognize both human and bovine leukocyte synthase, showing that these enzymes are immunologically similar.

In addition to human and bovine glycogen synthase (fig.1), the antisera recognized proteins in rabbit and rat liver and muscle homogenates (fig.2). The cross-reactivity between leukocyte and muscle glycogen synthase is in agreement with our previous findings that tryptic phosphopeptides (sites 1a, 1b and 2) from leukocyte and rabbit muscle glycogen synthase appear identical when analysed by HPLC [11]. The 90 kDa band found

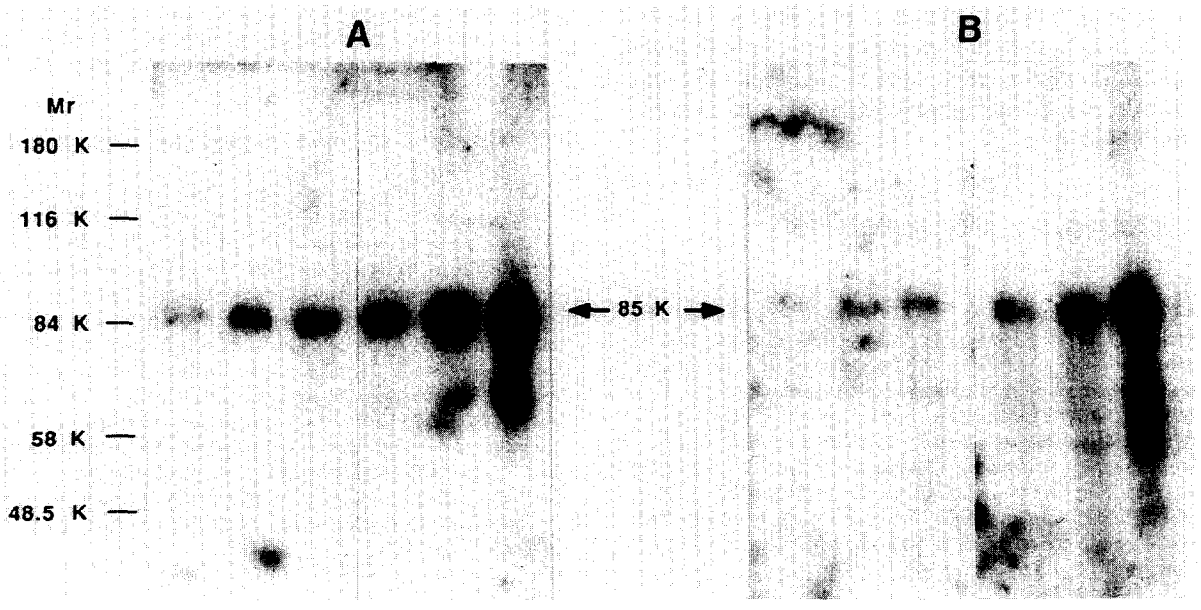

Fig.1. Western blot analysis of crude protein extracts from human leukocytes using rabbit antiserum against human leukocyte glycogen synthase (A) or rabbit antiserum against bovine leukocyte glycogen synthase (B). Increasing amounts of 17000 × *g* supernatant from human polymorphonuclear leukocytes were run in a 7.5% polyacrylamide gel and electrophoretically transferred to nitrocellulose as described in section 2. The amount of glycogen synthase in the crude cell extract was determined by the activity assay as described in section 2. The lanes contain (from left to right) 5, 10, 15, 25, 50 and 100 mU. Standard M_r markers were α-2-macroglobulin (180 kDa), β-galactosidase (116 kDa), fructose-6-phosphate kinase (84 kDa), pyruvate kinase (58 kDa) and fumarase (48.5 kDa).

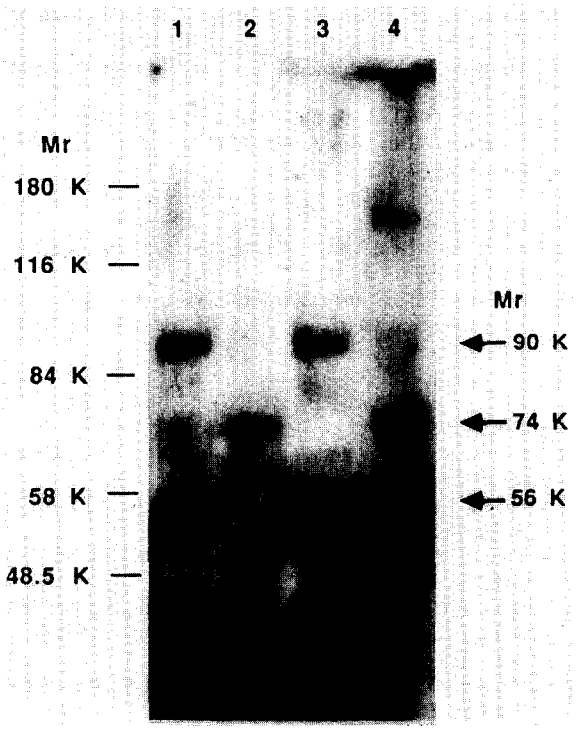

Fig.2. Western blot analysis of glycogen synthase in crude protein extracts from rabbit and rat muscle and liver. The SDS-polyacrylamide gel electrophoresis and immunoblotting were as described in section 2 and in the legend to fig.1. Lane 1, 17000 × *g* supernatant of rabbit muscle; lane 2, 17000 × *g* supernatant of rabbit liver; lane 3, 17000 × *g* supernatant of rat muscle; lane 4, 17000 × *g* supernatant of rat liver. The amount of glycogen synthase in the crude cell extracts was determined by the activity assay as described in section 2. Each lane contained 50 mU.

in the Western blot of crude rabbit and rat muscle extracts (fig.2, lanes 1 and 3) corresponds to the molecular mass of muscle glycogen synthase [23–25]. The 56 kDa band has been observed by others and found to contain a proteolytic fragment of the 90 kDa form [6].

Recent investigations of liver glycogen synthase revealed subunit sizes in the range 75–93 kDa [14,15]. Our antisera recognized predominantly a protein of 74 kDa in the crude liver extracts (fig.2, lanes 2 and 4). The occurrence of lower-M_r forms of glycogen synthase is considered to be due to partial proteolysis, for two reasons: (i) no protease inhibitors were added to the buffer used for homogenization of the rabbit and rat tissues and (ii) it has been shown that the glycogen synthase subunit is susceptible to proteolysis [9,15,26].

Because our antisera thus recognize glycogen synthase in liver and muscle, these tissues must contain an enzyme that resembles the leukocyte enzyme.

On the basis of immunochemical analysis using antisera against rat liver and skeletal muscle, Kaslow and co-workers have proposed the existence of two isozymes in these tissues. In Western blotting analysis, Kaslow found no immunological cross-reactivity and concluded that specific L-type (liver) and M-type (muscle) enzymes must exist [13,14]. However, other workers found that specific antibodies to rabbit skeletal muscle precipitate both rabbit and rat liver glycogen synthase in addition to the muscle enzyme [27,28]. Furthermore, it was found that monospecific antiserum to rat heart muscle glycogen synthase was able to recognize both rat heart and rat liver synthase [15]. These findings, together with our results from the study of leukocyte glycogen synthase, do not support the general hypothesis that L- and M-type enzymes can be distinguished by immunochemical analysis.

In any case, we must conclude that leukocyte glycogen synthase cannot be classified as either muscle (M-) or liver (L-) type glycogen synthase according to the nomenclature stated in [13,14]. However, differences have been found in the amino-acid sequences surrounding the sites which are recognized by cAMP-dependent protein kinase (sites 1a, 1b and 2), showing that glycogen synthase from rabbit muscle and liver are isozymes [12].

A similar study is needed for further classification of leukocyte glycogen synthase and for a better understanding of the heterogeneity of the enzyme. We are at present studying the amino acid sequences of phosphopeptides from leukocyte glycogen synthase.

Acknowledgements: We would like to thank Inger Bjerregaard for technical assistance and Lone Manner-Jakobsen and Lisbeth Heilesen for help in the preparation of the manuscript. This work was supported by grants from the Danish Medical Research Council, the Institute for Clinical Experimental Research, Aarhus University, Aarhus Universitets Forskningsfond, P. Carl Petersens Fond, Konsul Johannes Fogh-Nielsens og Fru Ella Fogh-Nielsens Legat, Arvid Nilssons Fond and Kong Christian den Tiendes Fond.

REFERENCES

[1] Roach, P.J. (1981) Curr. Top. Cell. Regul. 20, 45–105.
[2] Cohen, P. (1982) Nature 296, 613–620.
[3] Soderling, T.R. (1979) Mol. Cell. Endocrin. 16, 157–179.
[4] Preiss, J. and Walsh, D.A. (1981) in: Biology of Carbohydrates (Ginsburg, V. ed.) vol.1, pp.199–314, Academic Press, New York.
[5] Kuret, J., Woodgett, J.R. and Cohen, P. (1985) Eur. J. Biochem. 151, 39–48.
[6] Sheorain, S.V., Ramakrishna, S., Benjamin, W.B. and Soderling, T.R. (1985) J. Biol. Chem. 260, 12287–12292.
[7] Ahmad, Z., Lee, F., Depaoli-Roach, A. and Roach, P.J. (1984) J. Biol. Chem. 259, 8743–8747.
[8] Juhl, H., Sheorain, V.S., Schworer, C.M., Jett, M.F. and Soderling, T.R. (1983) Arch. Biochem. Biophys. 222, 518–526.
[9] Picton, C., Aitkin, A., Bilham, T. and Cohen, P. (1982) Eur. J. Biochem. 124, 37–45.
[10] Sølling, H. and Esmann, V. (1977) Eur. J. Biochem. 81, 119–128.
[11] Rasmussen, L.H., Pedersen, K.M. and Juhl, H. (1985) Biochimie 67, 615–623.
[12] Wang, Y., Bell, A.W., Hermodson, M.A. and Roach, P.J. (1986) J. Biol. Chem. 261, 16909–16915.
[13] Kaslow, H.R. and Lesikar, D.D. (1984) FEBS Lett. 172, 294–298.
[14] Kaslow, H.R., Lesikar, D.D., Antwie, D. and Tan, A.W.H. (1985) J. Biol. Chem. 260, 9953–9956.
[15] Rulfs, J., Wolleben, C.D., Miller, T.B. and Johnson, G.L. (1985) J. Biol. Chem. 260, 1203–1207.
[16] Thomas, J.A., Schlender, K.K. and Larner, J. (1968) Anal. Biochem. 25, 486–499.
[17] Juhl, H. (1981) Mol. Cell. Biochem. 35, 77–92.
[18] Pedersen, K.M., Rasmussen, L.H. and Juhl, H. (1985) Biochimie 67, 625–628.
[19] Bradford, M. (1976) Anal. Biochem. 72, 248–254.
[20] Laemmli, U.K. (1979) Nature 227, 680–685.
[21] Kyhse-Andersen, J.J. (1984) Biochem. Biophys. Methods 10, 203–209.
[22] Bjerrum, O.J. and Schäfer-Nielsen, C. (1986) in: Electrophoresis '86 (Dunn, M.J. ed.) pp.315–327, VCH, Weinheim.
[23] DePaoli-Roach, A., Ahmad, Z., Camici, M., Lawrence, J.C. jr and Roach, P.J. (1983) J. Biol. Chem. 258, 10702–10709.
[24] Lawrence, J.C. jr and James, C. (1984) J. Biol. Chem. 259, 7975–7982.
[25] Hiken, J.F. and Lawrence, J.C. jr (1985) Arch. Biochem. Biophys. 236, 59–71.
[26] Camici, M., DePaoli-Roach, A.A. and Roach, P.J. (1982) J. Biol. Chem. 257, 9898–9901.
[27] Ciudad, C., Camici, M., Ahmad, Z., Wang, Y., DePaoli-Roach, A.A. and Roach, P.J. (1984) Eur. J. Biochem. 142, 511–520.
[28] Ciudad, C., DePaoli-Roach, A. and Roach, P.J. (1984) Biochim. Biophys. Acta 804, 261–263.